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ORIGINAL ARTICLE

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Autologous serum supplement favours in vitro regenerative paracrine factors synthesis

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Abstract

Objectives: Foetal bovine serum (FBS) is often the serum supplement of choice for in vitro human cell culture. This study compares the effect of FBS and autologous human serum (AuHS) supplement in human peripheral blood mononuclear cell (PBMC) culture to prepare secretome.

Materials and methods: The PBMC (n = 7) were cultured either in RPMI-1640 containing L-glutamine and 50 units/ml Penicillin-Streptomycin (BM) or in BM with either AuHS or FBS. Viability, proliferation and differentiation of PBMC were evaluated. Paracrine factors present in the secretomes (n = 6) were analysed using ProcartaPlex Human Cytokine panel (17 plex). Ingenuity Pathway Analysis (IPA) was performed to predict activation or inhibition of biological functions related to tissue regeneration.

Results: The viability of PBMC that were cultured with FBS supplement was significantly reduced at 96 h compared to those at 0 and 24 h ($P < .05$). While the reduction of the viability of PBMC that were cultured with AuHS supplement was not significantly different compared to those at 0 and 24 h. The FBS secretomes prepared at 24 h was found to contain significantly higher amount of EGF ($P < .05$) compared to that in AuHS or BM secretome. The AuHS secretomes contained significantly higher amount of HGF at 24 ($P < .05$) and 96 h ($P < .01$), and VEGF-A at 24 h ($P < .05$) compared to those in the FBS secretomes. SDF-1 was not detected in the FBS secretomes prepared at either 24 or 96 hours. Double immunocytochemical staining revealed a marked increase in co-localization of SDF-1 and its receptor in PBMC that were cultured with AuHS supplement compared to that cultured with FBS supplement.

Conclusion: In secretome preparation, AuHS supplement favours synthesis of paracrine factors that are needed for regenerative therapy.

1 | INTRODUCTION

The regenerative potential of the secretomes of stem and progenitor cells has been reported to treat neuronal disorders,[1] vascular diseases[2] and cutaneous wounds.[3] The secretome used for regenerative medicine are generally prepared using different types of human cells such as adult stem cells,[4] freshly isolated healthy peripheral blood mononuclear cells (PBMC)[5,6] and apoptosis induced PBMC.[7–9]

In the preparation of secretome, foetal bovine serum (FBS) is commonly used as the serum supplement in cell culture. As a rich source of

Abbreviations: AuHS, autologous human serum; BDNF, brain-derived neurotrophic factor; Creb, cAMP response element-binding protein; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinases; FAK, focal adhesion kinase; FBS, foetal bovine serum; Fcer1, high-affinity IgE receptor; FGF-2, fibroblast growth factor 2; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HGF, hepatocyte growth factor; HMGB1, high-mobility group box 1 protein; HSP, heat shock protein; IL, Interleukin; IPA, Ingenuity Pathway Analysis; Jnk, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; M-CSF, macrophage colony stimulating factor; NFκB, nuclear factor kappa B; PBMC, peripheral blood mononuclear cells; PDGF-BB, platelet-derived growth factor beta; SCF, stem cell factor; SDF-1a, stromal cell-derived factor-1a; VEGF-A, vascular endothelial growth factor A.

proteins, growth factors, hormones, lipids, vitamins, attachment factors and other important trace elements, FBS supports the survival and proliferation of cells during in vitro cell culture.[10] However, the composition of FBS varies from lot-to-lot. Again, Neu5GC present in FBS may trigger immune response in xenogeneic culture such as human cells.[11] As an alternative, serum-free supplements such as purified or recombinant proteins are used to produce secretome.[5–9] Compared to FBS, serum-free supplements maintain better safety, reproducibility and consistency of cells during in vitro culture.[12,13] Nonetheless, the recombinant or purified protein supplements were known to regulate the composition of paracrine factors in secretome by modulating the autocrine and paracrine signalling pathways.[14]

As an alternative to either FBS or serum-free supplements, the present study analysed the potential of autologous human serum (AuHS) for the production of regenerative paracrine factors in the secretome. The serum of a donor was used as the supplement to culture the PBMC, which were isolated from the blood of the same individual. Parallel cultures were maintained with either FBS supplement or without any serum supplement to compare the potential advantage of using AuHS.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

In this study, blood samples were collected after acquiring informed written consents from the donors. The sample collection procedure was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Reference #DF RD1301/0012(L)].

2.2 | Preparation of serum and isolation of PBMC

Blood was collected from healthy male donors, aged 21–35 y (n = 7) having no history of: smoking, alcohol consumption, drug and/narcotics addiction, diagnosis of any inflammatory diseases either chronic or at least in the last 4 weeks, major surgical treatment in the last 1 y, and immunotherapy. Blood was collected from each donor in two steps: (i) 20 ml without any anticoagulant for serum preparation, and (ii) 30 ml in vacutainer containing sodium heparin (GmbH, Hamburg, Germany) for PBMC isolation.

To prepare the serum, 20 ml of blood was transferred to a 50 ml centrifuge tube (BD Bioscience, Franklin Lakes, NJ, USA) and was left at room temperature for an hour to facilitate coagulation. Then the tube containing the coagulated blood was centrifuged at 400 × g for 15 min to collect crude serum in sterile centrifuge tube for a second round of centrifugation at 1800 × g for 15 min to remove cell debris or insoluble particles. The final serum supernatant was heat treated at 57 ± 2°C for 30 min to inactivate complement and used as AuHS supplement in the respective PBMC cultures.

To isolate PBMC, 10 ml of blood was transferred into a 50 ml centrifuge tubes (BD Bioscience) and diluted with equal volume of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Gibco, Grand Island, NY, USA). The diluted blood was then added into another 50 ml centrifuge tube (BD Bioscience) containing 15 ml of Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and centrifuged at 400 × g for 30 min with brake off. Buffy coat containing PBMC was collected and washed twice with DPBS by centrifugation at 200 × g for 10 min. After discarding the supernatant, the cell pellet was mixed with basal media (BM) consists of RPMI-1640 containing L-glutamine (Gibco) and 50 units/ml Penicillin-Streptomycin (Gibco). Cell number was counted using Trypan Blue (Gibco) dye exclusion method.

2.3 | Media preparation and culture

Isolated PBMC were cultured either in BM, or in BM with either 10% (v/v) FBS (Gibco) or 10% (v/v) freshly prepared AuHS supplement. PBMC were seeded in 12-well plates at a density of 5.5×10^5 cells/ml in different media. The plates were stored at 37°C and 5% CO_2 in humidified chambers, and incubated up to 96 h.

2.4 | Cell viability assay

The number of live PBMC was counted using Trypan Blue (Gibco) dye exclusion method at every 24 h interval until 96 h. The number of live cells was recorded as mean percentile (±SE) of increase or decrease in respect to the initial seeding number.

2.5 | Preparation of cytospin slides of PBMC

After 24 and 96 h of initial incubation, PBMC were harvested, washed twice using DPBS (Gibco) and the pellets were resuspended in 10% FBS (Gibco) containing DPBS (Gibco) to obtain 5×10^5 cells/ml. PBMCs were attached on poly-L-lysine coated slides (Thermo Scientific Shandon, Cheshire, UK) using cytocentrifuge (Thermo Scientific Shandon) and dried overnight in desiccation chamber followed by fixation in ice cold absolute acetone (Sigma-Aldrich, Steinheim, Germany) for 5 minutes and drying at room temperature for 10–15 min.

2.6 | Differential count of PBMC

Acetone fixed air dried slides were stained using Giemsa stain (Sigma-Aldrich) for differential count. In brief, the slides were stained with 5% Giemsa stain for 20 min; washed briefly using deionized water and left in vertical position to air dry at room temperature. The number of myeloid and lymphoid cells were counted using microphotographs taken by axiocam ERc5s (Carl Zeiss, Jena, Germany) attached with microscope (Carl Zeiss). Three randomly chosen fields from each slide were chosen for microphotographs and subsequent cell counting. Differential count was presented as mean % of total cells ± SE.

2.7 | Paracrine factors profiling

Culture supernatants were harvested at 24 and 96 h to profile the composition of paracrine factors present therein. Luminex-based ProcartaPlex human cytokine/chemokine 17plex immunoassay kit from e-Bioscience (affymetrix, e-Bioscience, Vienna, Austria) was used to analyse the presence or absence of 17 selected paracrine

factors known to either stimulate or inhibit proliferation, migration and differentiation of different cell types including PBMC (Table S1).

2.8 | Immunocytochemistry

Cyotspin slides prepared using PBMC harvested at 24 hours were used for immunocytochemical staining of CXCR-4 and SDF-1. Anti-human monoclonal CXCR-4 antibody raised in mouse (Santa Cruz sc-53534, TX, USA) and anti-human polyclonal SDF-1 antibody raised in rabbit (Santa Cruz sc-28876) were used as sources of primary antibodies. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Santa Cruz sc-2010) and peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5) conjugated anti-rabbit IgG (Santa Cruz sc-45101) were used as secondary antibodies. Acetone (Sigma-Aldrich) fixed air dried slides were treated with dilution buffer [DPBS (Gibco) containing 10% FBS (Gibco)] and incubated at room temperature for 30 min to block unspecific binding. The slides were then treated as follows: washed three times with DPBS (Gibco); incubated overnight with 100× diluted primary antibodies at 4°C in humidified chamber; washed three times with DPBS; incubated with the 200× diluted secondary antibodies for one hour at room temperature in dark; washed three times with DPBS; and finally, counter stained with Fluoroshield with DAPI (Sigma) for nuclear staining.

2.9 | Molecular network analysis

The corresponding Entrez Gene ID of the selected 17 paracrine factors and the fold changes of their up-regulated and down-regulated expression in BM and AuHS secretome in respect to those in FBS secretome were imported into the core analysis tool of Ingenuity Pathway Analysis (IPA) (Ingenuity systems; www.ingenuity.com). The IPA was performed to predict activation or inhibition of biological functions and signalling pathways involved in proliferation, migration, differentiation and apoptosis. If any paracrine factor was not expressed, the lowest detection limit of multiplex analyser (Luminex, TX, USA) for the respective paracrine factor was used as its expression value. Positive fold change values denote up-regulation, and negative values denote down-regulation. Activation and inhibition of biological functions were predicted by the 'z-score' calculated by IPA on the basis of the expression pattern of downstream transcriptional regulator in IPA software. Positive and negative z-score indicates activation and inhibition of biological function, respectively. The z-score greater than +1.96 or smaller than −1.96 were considered significant ($P < .05$).

The IPA was further performed to compare the most contributing functional network(s) involving the paracrine factors analysed in the study in the secretome of AuHS prepared at 24 and 96 h. IPA utilizes Fisher's exact test indicate significant association ($P = .05$).

2.10 | Data analysis

Except for molecular network analysis, all other data were analysed using ANOVA and Tukey's HSD post-hoc (SPSS version 22). The significant level was set at $P < .05$.

3 | RESULTS

3.1 | Cell viability

At 24 h of incubation, the number of viable PBMC in FBS and AuHS supplemented media was increased by ~60% and ~70%, respectively, compared to initially seeded viable cells. It was observed that with time, the rate of increase of viable PBMC in FBS supplemented media was lower, however, only the 96 h post-incubation was significantly different from 24 and 48 h ($P < .05$). While in the AuHS supplemented media there was no significant difference in the rate of increase of viable PBMC between all incubation time frame. The number of live PBMC cultured in BM was gradually decreasing and was reduced by 70% at 96 h with respect to the initial count (Figure 1A). The

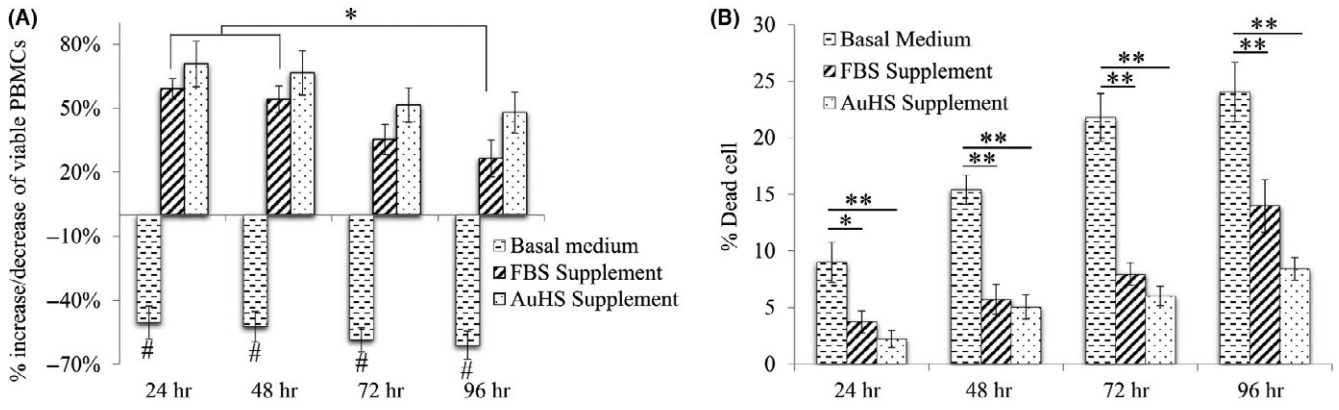

FIGURE 1 Effect of serum supplement on the viability of peripheral blood mononuclear cells (PBMC). (A) Rate of increase of live PBMC did not differ significantly in autologous human serum (AuHS) supplemented media until 96 h. While, in foetal bovine serum (FBS) supplemented media, rate of increase of viable PBMC at 96 h was significantly different from that at 24 and 48 h. In basal medium (BM), the number of live PBMC gradually decreased and was reduced by 70% of the initial count at 96 h. (B) The percentage of the dead cells was significantly lower in FBS and AuHS supplemented media compared to that in BM. [#, significantly different ($P < .01$) compared to both FBS and AuHS groups; *, $P < .05$; **, $P < .01$; $n = 7$]

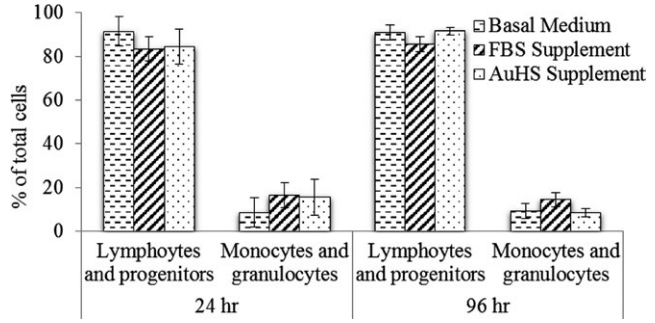

FIGURE 2 Effect of serum supplement on the differentiation of peripheral blood mononuclear cells (PBMC). Differences in serum supplement did not show any effect on the differential count of PBMC until 96 h. (AuHS, autologous human serum; FBS, foetal bovine serum)

percentage of dead PBMC was significantly lowered in AuHS and FBS supplemented media compared to that in BM at all incubation time frame (Figure 1B).

3.2 | Differentiation

On the basis of morphology, PBMC were divided into two groups: (i) lymphocytes and progenitors, and (ii) monocytes and granulocytes (Figure S1). The percentages of different types of cells at 24 and 96 h in BM, AuHS supplemented media and FBS supplemented media did not differ significantly (Figure 2).

3.3 | Paracrine factors profile in the secretomes

Seventeen paracrine factors were analysed in the secretomes at 24 and 96 h of incubation. The detected level of paracrine factors varied from one secretome to another (Table S2).

3.4 | Comparative paracrine factors expression in the secretomes

3.4.1 | AuHS vs FBS secretome

At 24 h, significantly higher expressions of HGF ($P < .05$) and VEGF-A ($P < .05$) were detected in the AuHS secretomes compared to those in the FBS secretomes (Figure 3). The HGF expression at 96 h was significantly higher in the AuHS secretomes compared to that in the FBS secretomes ($P < .01$) (Figure 3). Whereas the expression of EGF ($P < .05$) was significantly higher in the FBS secretomes when compared to the AuHS secretomes at 24 h (Figure 3). Notably, SDF-1A was detected in the AuHS secretomes but not in the FBS secretomes either at 24 or 96 h (Figure 3).

3.4.2 | AuHS vs BM secretome

At 24 h, expressions of BDNF ($P < .01$), G-CSF ($P < .01$), PDGF-BB ($P < .05$) and SDF-1A ($P < .01$) were significantly higher in the AuHS secretomes compared to those in the BM secretomes (Figure 3). At 96 h, expressions of BDNF ($P < .01$), G-CSF ($P < .05$) and HGF

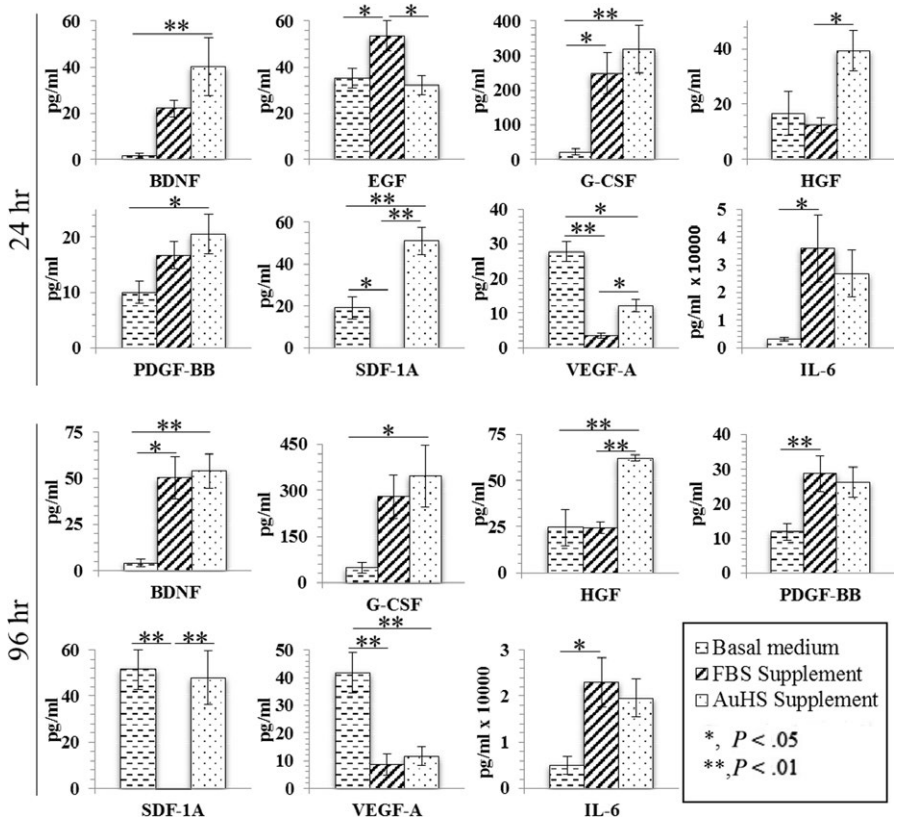

FIGURE 3 Comparison of the amount of paracrine factors in the secretomes of peripheral blood mononuclear cells (PBMC). Difference in the amount of paracrine factors namely, brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), platelet-derived growth factor beta (PDGF-BB), stromal cell-derived factor-1A (SDF-1A), vascular endothelial growth factor A (VEGF-A) and interleukin 6 (IL-6) in the secretomes prepared from PBMC that were cultured with either autologous human serum (AuHS), foetal bovine serum (FBS) or without any serum supplement i.e., in basal medium (BM) at 24 h and 96 h of incubation. (* = $P < .05$, ** = $P < .01$, $n = 6$)

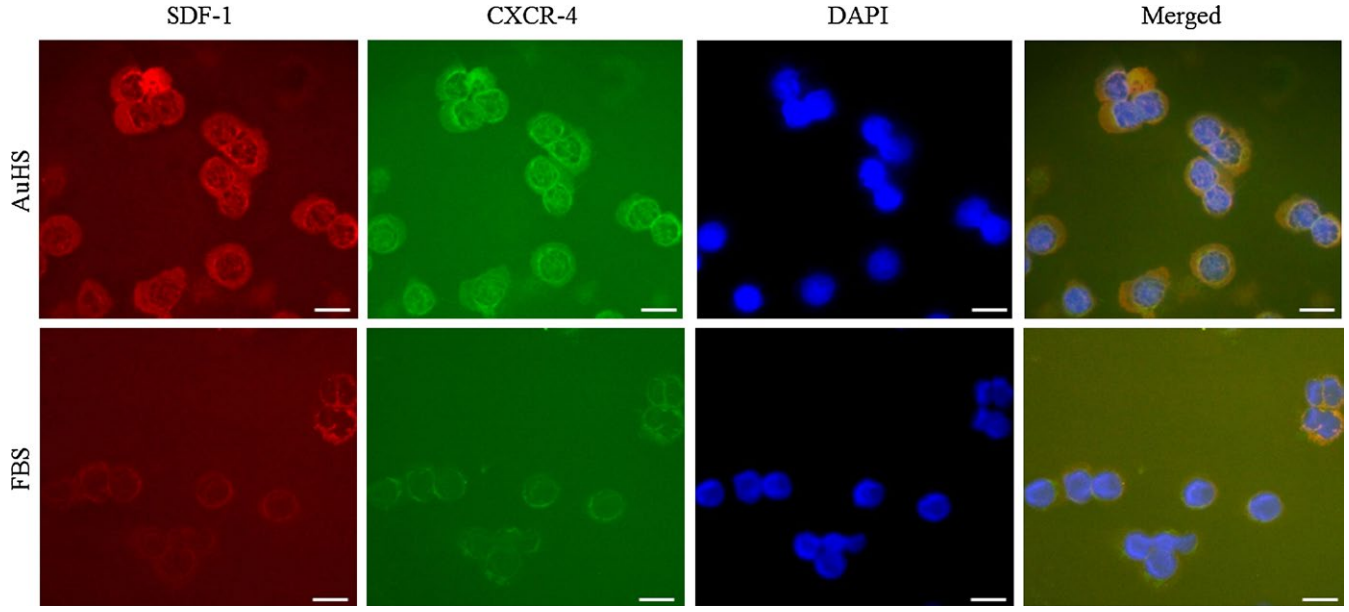

FIGURE 4 Fluorescent immunostaining of CXCR-4 and stromal cell-derived factor-1A (SDF-1A) in peripheral blood mononuclear cells (PBMC) that were cultured in autologous human serum (AuHS) and foetal bovine serum (FBS) supplemented media. CXCR-4 and SDF-1 was detected using mouse anti-human monoclonal and rabbit anti-human polyclonal antibodies, respectively. Fluorescein isothiocyanate (FITC, green) and peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5, red) conjugated with goat anti-mouse and goat anti-rabbit IgG were used as secondary antibodies. 4′,6-diamidino-2-phenylindole (DAPI) was used to perform nuclear counter staining. A marked increase of colocalized SDF-1 and it's receptor CXCR-4 is visible in the PBMC cultured in AuHS supplemented media. (Scale bar: 10 μm)

($P < .01$) were significantly higher in the AuHS secretomes compared to those in the BM secretomes (Figure 3). Whereas, only VEGF-A expression was significantly higher in the BM secretomes compared to that in the AuHS secretomes both at 24 ($P < .05$) and 96 h ($P < .01$) (Figure 3).

3.4.3 | FBS vs BM secretome

At 24 h, the expression of EGF ($P < .05$), G-CSF ($P < .05$) and IL-6 ($P < .05$) were significantly higher in the FBS secretomes compared to those in the BM secretomes (Figure 3). At 96 h, the expression of BDNF ($P < .05$), PDGF-BB ($P < .01$) and IL-6 ($P < .05$) were significantly higher in the FBS secretomes compared to those in the BM secretomes (Figure 3). The expression of VEGF-A in the BM secretomes was significantly higher ($P < .01$) compared to that in the FBS secretomes both at 24 and 96 h (Figure 3). Unlike in FBS secretomes, SDF-1A was detected in BM secretomes both at 24 or 96 h of incubation (Figure 3).

3.5 | Immunocytochemistry

A markedly higher expression of both SDF-1 and its receptor, i.e., CXCR-4 was detected in the PBMC that were cultured in AuHS supplemented media compared to that cultured in FBS supplemented media (Figure 4). Prominent colocalization of SDF-1 and its receptor CXCR-4 was also detected in the PBMC cultured in AuHS supplemented media (Figure 4).

3.6 | Relative expression (mean fold changes) of paracrine factors in AuHS and BM secretomes compared to the FBS secretomes

At 24 h, 13 paracrine factors (BDNF, G-CSF, GM-CSF, HGF, LIF, M-CSF, PDGF-BB, SCF, SDF-1A, VEGF-A, IL-2, IL-3 and IL-12p70) were expressed more in the AuHS secretomes, and 9 paracrine factors (FGF-2, HGF, LIF, M-CSF, SCF, SDF-1A, VEGF-A, IL-3 and IL-23) were expressed more in the BM secretomes compared to the respective factors expressed in the FBS secretomes. At 96 hours, a lower level of IL-6, EGF and PDGF-BB were detected in the AuHS secretomes compared to those in the FBS secretomes. Expression of 11 paracrine factors (FGF-2, GM-CSF, HGF, LIF, M-CSF, SCF, SDF-1A, VEGF-A, IL-2, IL-3 and IL-23) in the BM secretomes were higher than those in the FBS secretome at 96 h (Table S3).

3.7 | Biological functions regulated by paracrine factors secreted from human PBMC

IPA was performed to identify the most significant molecular networks and signalling pathways relevant to these paracrine factors, and their role in maintaining biological functions. Ten most important biological functions related to proliferation, viability, apoptosis and differentiation, were selected (Figure 5). Predicted 'z-score' of the functions showed that the paracrine factors of the AuHS secretomes prepared at 24 and 96 h promote proliferation, viability, and differentiation while inhibiting apoptosis. Significant

Biological Functions (p value)	Cytokines analyzed																	Activation (↑) Inhibition(↓)			
																		AuHS		BM	
	BDNF	EGF	FGF2	G-CSF	GM-CSF	HGF	LIF	M-CSF	PDGF-BB	SCF	SDF-1A	VEGF-A	IL-2	IL-3	IL-6	IL-12	IL-23	24 hr	96 hr	24 hr	96 hr
Proliferation of immune cells (1.86E-14)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	√	√	↑	↑	-	-
Proliferation of hematopoietic progenitor cells (6.39E-14)	-	-	-	√	√	-	-	-	-	√	√	-	√	√	√	-	-	-	-	-	-
Cell viability of leukocytes (1.50E-19)	-	√	-	√	√	√	-	√	-	√	√	-	√	√	√	-	-	↑	↑	-	↑
Cell viability of mononuclear cells (4.51E-17)	-	√	-	-	√	√	-	√	-	√	√	-	√	-	√	-	-	↑	↑	-	↑
Apoptosis of leukocytes (2.32E-12)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	-	-	↓	↓	-	-
Apoptosis of hematopoietic progenitor cells (2.23E-10)	-	-	-	√	√	-	-	-	-	√	-	-	-	√	√	-	-	↓	↓	-	-
Differentiation of leukocytes (4.07E-18)	-	-	-	√	√	√	√	√	-	√	-	-	√	√	√	-	√	-	↑	-	-
Differentiation of hematopoietic progenitor cells (2.83E-14)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	-	-	-	-	-	-
Quantity of leukocytes (8.75E-11)	-	-	-	√	√	-	-	-	-	√	-	-	√	√	√	-	-	↑	↑	-	-
Quantity of hematopoietic progenitor cells (8.34E-14)	-	-	-	√	√	-	-	-	-	√	-	-	√	√	-	-	-	↑	↑	-	-

Activation (↑) and inhibition (↓) were measured by the positive and negative z-scores (Table S2). z-score > +1.96 or < -1.96 were considered significant (n=6). [√, detected; -, unchanged or not detected]

FIGURE 5 Predicted activation or inhibition of biological functions maintained by the 17 paracrine factors that were analysed in the autologous human serum (AuHS) and basal medium (BM) secretomes compared to the foetal bovine serum (FBS) secretome

activation or inhibition of any of those biological functions could not be predicted from the detected composition of paracrine factors of the BM secretomes prepared at 24 h compared to those of the FBS secretomes. However, significant activation of viability of leukocytes and mononuclear cells was predicted in the BM secretomes prepared at 96 h compared to that in the FBS secretomes (Figure 5).

3.8 | Activation of high-mobility group box 1 protein (HMGB1) signalling pathway

IPA was also performed to identify the top most affected signalling pathways that could be influenced by the 17 paracrine factors analysed in the study. The top 10 pathways being affected (most to least) by the analysed paracrine factors are listed in Table 1. IPA predicted

TABLE 1 Top 10 signalling pathways identified by IPA that could be regulated by 17 analysed paracrine factors

No.	Pathways	−log *P*[a]	Ratio[b]
1	Haematopoiesis from pluripotent stem cells	1.96E01	2.05E-01
2	Haematopoiesis from multipotent stem cells	1.57E01	5E-01
3	Role of cytokines in mediating communication between immune cells	1.38E01	1.35E-01
4	Differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F	1.15E01	2.78E-01
5	Altered T cell and B cell Signalling in rheumatoid arthritis	1.02E01	7.41E-02
6	Hepatic fibrosis/hepatic stellate cell activation	9.62E00	3.57E-02
7	HMGB1 signalling	9.16E00	5.08E-02
8	Role of pattern recognition receptors in recognition of bacteria and viruses	9.13E00	5.04E-02
9	Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	8.46E00	2.44E-02
10	Hepatic cholestasis	8.39E00	3.8E-02

[a]Higher − log *P* value denotes higher chance of being regulated by the paracrine factors analysed in this study.

[b]The ratio indicates the dividend of presence of the analysed paracrine factors to the total number of paracrine factors involved in the corresponding pathway.

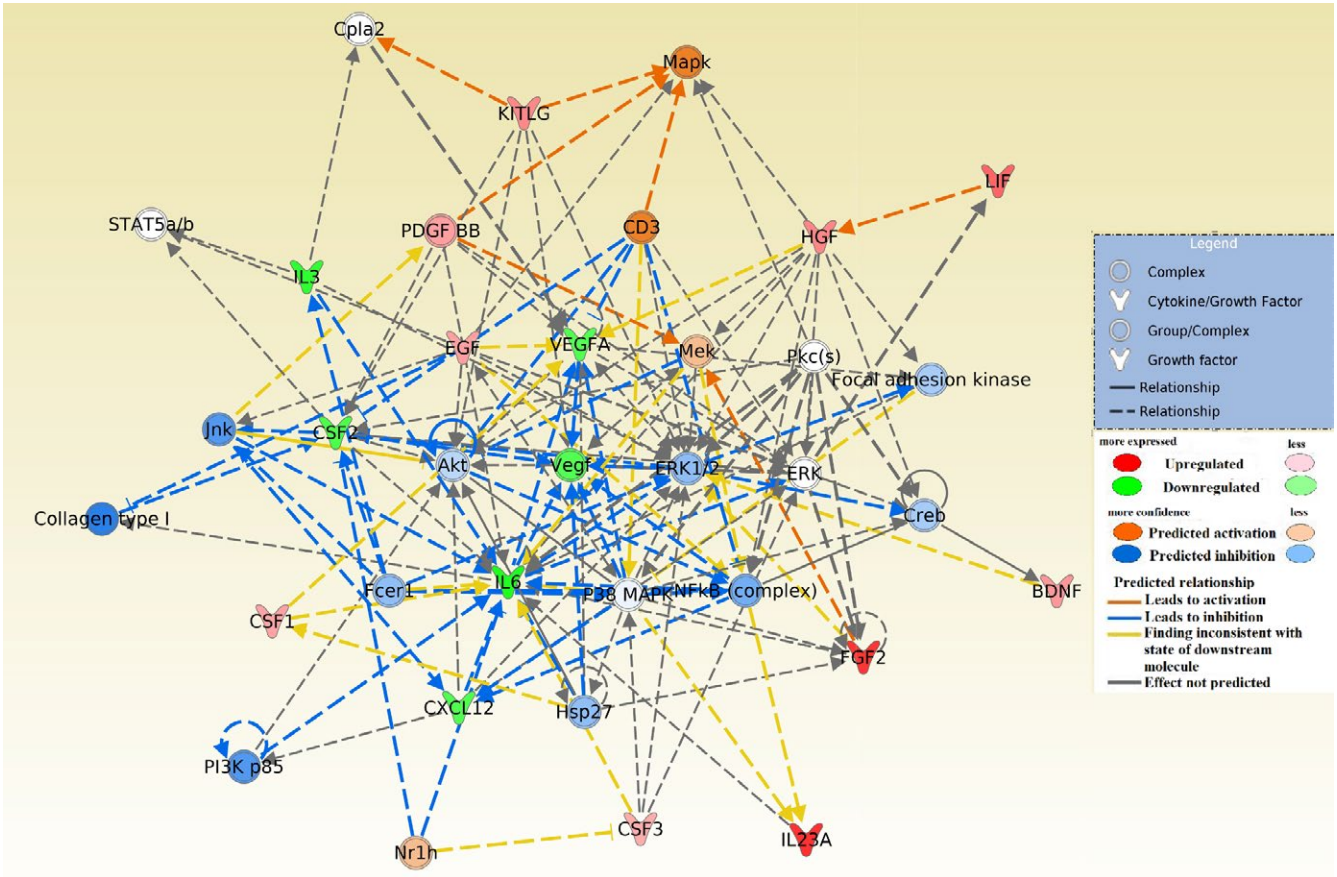

FIGURE 6 Illustration of the predicted ranked 1 functional network involving the paracrine factors of the autologous human serum (AuHS) secretome that were detected at 24 h in comparison to those detected at 96 h. Functional pathway predicted mild inhibition of nuclear factor kappa B (NFκB), extracellular-signal-regulated kinases (ERK) 1/2, focal adhesion kinase (FAK), AKT, heat shock protein (HSP) 27, high-affinity IgE receptor (Fcer1) and cAMP response element-binding protein (Creb), and strong inhibition of the c-Jun N-terminal kinase (Jnk), P13KP85 and Collagenase type 1 in the AuHS secretome prepared at 96 h compared to that of 24 h. Meanwhile, mild activation of MEK and Nr1 h; and strong activation of MAPK and CD3 have been predicted in the AuHS secretomes prepared at 96 h

that the AuHS secretomes prepared both at 24 and 96 hours have the potential to activate HMGB1 signalling pathway (z = 1.633) more than that of the FBS secretomes.

3.9 | Functional molecular networks of paracrine factors secreted from human PBMC

IPA was further performed to analyse the functional molecular network involving the paracrine factors selected in the study. The ranked 1 (P = 1.00E-33) functional molecular network connecting cell-to-cell signalling and interaction, cellular growth and proliferation, and cellular development was shown to involve 15 out of the 17 paracrine factors analysed in this study (Figure 6). The ranked 1 functional network was further analysed (Figure 6) to check the predicted activation or inhibition of the downstream molecules involved in the network using the expression profile of the paracrine factors detected in the AuHS secretome prepared at 24 and 96 h (Table S4). A mild inhibition was predicted for nuclear factor kappa B (NFκB), extracellular-signal-regulated kinases (ERK) 1/2, focal adhesion kinase (FAK), AKT, heat shock protein (HSP) 27, high-affinity IgE receptor (Fcer1) and cAMP response element-binding protein (Creb) in the AuHS secretome prepared at 96 h compared to that prepared at 24 h. Strong inhibition was predicted for c-Jun N-terminal kinase (Jnk), P13KP85 and Collagenase type 1 in the AuHS secretome prepared at 96 h. Meanwhile, mild activation of MEK and Nr1 h, and strong activation of MAPK and CD3 was predicted in the AuHS secretome prepared at 96 h (Figure 6).

4 | DISCUSSION

Composition of paracrine factors in secretomes largely depends on the initial media composition as well as the type of cultured cells. Animal serum, especially FBS, has been widely used as media supplement, as it provides proteins, growth factors, hormones, lipids, vitamins and other important trace elements in culture.[10] FBS, of which the exact composition is not well-defined, can be a vehicle of disease transmission, and can trigger immune response for its Neu5GC antigen.[11,12] Serum free media supplemented with recombinant human protein or purified animal serum proteins have been used as an alternative to FBS in cell culture.[11,12] However, a number of purified or

recombinant proteins given as supplement in serum free media were shown to affect paracrine secretions that in turn might exert feedback inhibition on the proteins.[14] In the search for another alternative, this study investigated the potential of AuHS in producing secretome while maintaining proliferation and viability of PBMC in vitro.

Human sera were collected from individuals who did not have history of smoking, alcohol consumption, drug or narcotics addiction, chronic inflammatory diseases; major surgical treatment in the last 1 y; or immunotherapy for the preparation of AuHS. It was reported that smoking and alcohol consumption affected the expression of a number of paracrine factors such as IL-1β, IL-8, IL-12, MCP-1, VEGF.[15,16] The other exclusion criteria are directly linked with the changes in immunological and inflammatory responses which will result in the change of the paracrine factor expressions in the circulation of the host.

To evaluate the impact of AuHS and FBS in secretome preparations, 17 paracrine factors commonly known to regulate proliferation, migration and differentiation related to tissue regeneration, were analysed in the respective secretomes (Table S1). However, the paracrine factors were analysed only at 24 and 96 h in the secretomes prepared using PBMC. It is well-known that the in vivo or in vitro half-life of any paracrine factor generally do not exceed more than a couple of hours.[17–19] Therefore, it was expected that the paracrine factors present in the sera at the time of separation would have minimum or no effect at 24 and 96 h of in vitro culture. Hence, the effects of the AuHS and FBS secretomes reported in the study would present the effects of the paracrine factors secreted during in vitro culture.

The higher in vitro PBMC viability in the AuHS supplemented media compared to that in the FBS supplemented media (Figure 1) might be linked with the secretome composition, i.e., higher amount of HGF, VEGF-A and SDF-1A as well as lower amount of EGF. The FBS secretomes that were prepared at 24 h had higher EGF compared to that of the AuHS secretomes and the BM secretomes. In the AuHS secretomes, a significantly higher expression of HGF and SDF-1A (at 24 and 96 h), and VEGF-A (at 24 h) were detected compared to those in the FBS secretomes (Figure 3). Both HGF and VEGF-A are known to play important roles in angiogenesis, cell proliferation, anti-apoptosis, chemotaxis, cell growth and differentiation.[20–22] Again, SDF-1A that was found in the AuHS secretomes, is known to play important roles in cell migration, cellular differentiation, cell cycle regulation and survival.[23] Expressions of SDF-1 and its receptor CXCR-4 were further confirmed by double immunostaining (Figure 4) and the result confirmed an increased expression as well as co-localization of both markers in PBMC cultured with the AuHS supplement compared to that cultured with the FBS supplement.

Butler et al. (2005) reported that patients with diabetic macular oedema had an average of 75 pg/ml SDF-1 in their vitreous, whereas in the vitreous of control nondiabetic patient SDF-1 was not detected.[24] Primary cultures of endothelial cells also expressed 50–130 pg/ml of SDF-1 in their secretome collected at 2, 24, 48 and 72 h of incubation.[25] In another in vivo study, ~40 pg/ml of SDF-1 in the plasma of the control hamster was observed, while ~100 pg/ml of SDF-1 was detected in the plasma of the animals treated with intramuscular VEGF.[26] In this study, 50–60 pg/ml of SDF-1A was detected in the both AuHS secretomes prepared at 24 and 96 h. This results are similar to those reported in the above-mentioned studies, hence we deduced that it could be adequate to initiate paracrine reactions.

Like AuHS secretome, SDF-1A was expressed in BM secretome as well. At 24 h of incubation the expression of SDF-1A in BM secretome (~20 pg/ml) was significantly ($P < .01$) lower than that of AuHS secretome (~50 pg/ml) (Figure 3). This could be linked to the significantly lower number of viable cells in BM compared to AuHS supplemented media (Figure 1). Despite significantly lower number of viable cells, at 96 h SDF-1A expression in BM secretome was similar to AuHS secretome (Figure 3). Intracellular expression of SDF-1A in monocytes and lymphocytes has been reported in several studies.[27,28] In BM, percentage of dead cells was significantly higher than that of AuHS (Figure 1B). Hence, the presence of SDF-1A in BM secretome at 96 h could be secreted or released from the pool of live and dead cells, respectively. Along with poor cell viability (Figure 1), the composition of the secretome in BM was not superior compared to FBS or AuHS in term of paracrine factor composition (Table S2) and predicted biological functions (Figure 5). It is noteworthy to mention that SDF-1A in BM secretome at 24 h was significantly lower than that of AuHS secretome. Hence, immunostaining of PBMC cultured in BM was excluded.

Lichtenauer et al. (2011) reported the presence of VEGF, HGF, FGF-2, SDF-1, G-CSF and GM-CSF in both the viable and the induced apoptotic PBMC secretomes harvested at 24 h.[7] Consistently, similar paracrine factors were detected in the PBMC secretomes harvested from AuHS supplemented media. In the current study, significantly higher expressions of G-CSF ($P < .01$) was found in the AuHS secretomes compared to the BM secretomes (Figure 3) at 24 h. While, at 96 h, the expression of G-CSF ($P < .05$) and HGF ($P < .01$) were significantly higher in the AuHS secretomes compared to those in the BM secretomes (Figure 3). Meanwhile, only VEGF-A expression was significantly higher in the BM secretomes compared to that in the AuHS secretomes both at 24 ($P < .05$) and 96 h ($P < .01$). Hoetzenecker et al. (2013) reported reduced amount of proinflammatory cytokine IL-6 in blood plasma of PBMC secretome treated animals.[6] Accordingly, the current study showed IL-6 expression in secretomes in the order of FBS>AuHS>BM.

To elucidate the effect of serum supplement on the differentiation of PBMC, analysis of differential count was performed. The effects of AuHS and FBS supplementation on PBMC differentiation during in vitro culture are not significantly different (Figure 2). However, at 96 h the percentage of lymphoid cells and progenitors were slightly higher in PBMC cultured with AuHS supplementation compared to that of FBS supplementation (Figure 2). Besides, percentage of dead PBMC was also lower in AuHS supplemented culture compared to that of FBS supplemented culture (Figure 1B). These results complement the notion that AuHS might offer additional advantage to maintain the lymphoid development and differentiation, and inhibit apoptosis of PBMC, most likely due to the presence of paracrine factors especially SDF-1 in the secretome.[29]

Prediction on the biological function analysis (Figure 5) also supports the in vitro PBMC viability and proliferation in the AuHS supplemented media (Figure 1). Therefore, it is expected that AuHS

secretome might favour in vivo regeneration of organ by increasing proliferation, viability, migration and homing of stem cells and progenitor cells (Table S5). Besides the potential use in the regeneration of damaged organs, secretome rich in SDF-1 might also help the homing of transplanted hematopoietic stem cells in bone marrow.[30]

Prediction on activation of signalling pathway showed that the AuHS secretomes favour activation of HMGB1 signalling pathway more than that by the FBS secretomes. HMGB1 plays a key role in maintaining tissue homeostasis, cleaning damaged and infected tissues, protecting non-injured tissues, and accelerating the process of regeneration[31] of tissues such as skeletal muscle,[32,33] epithelial[31] and spinal cord.[34] In 2012 Schiraldi et al. reported the role of SDF-1 and CXCR-4 in the HMGB1 mediated inflammatory cell recruitment in the site of damaged tissue[35] which is vital for tissue repair and regeneration. Studies reported the induced expression of CXCR-4 by G-CSF,[36] SCF and IL-6[37] in the hematopoietic stem cells. Expression of these paracrine factors in the AuHS and FBS secretome may be involved in the expression of CXCR-4 in the PBMC cultured in both AuHS and FBS supplemented media (Figure 4). Nonetheless, higher expression of G-CSF and SCF (Table S3) in AuHS secretome might be the cause behind higher expression of CXCR-4 receptor in PBMC cultured in AuHS medium compared to cells cultured in FBS medium. Moreover, distinctly higher expression of SDF-1 was observed in PBMC cultured in AuHS medium compared to that in FBS medium (Figure 4).

Although the advantage of using the AuHS secretome over the FBS secretome was apparent, the required time of incubation to prepare suitable AuHS secretome was inconclusive. This is mainly because the AuHS secretomes prepared at either 24 or 96 h exhibited equal potential in viability (Figure 1) and differentiation (Figure 2). Hence, the functional network analysis was performed using IPA. A mild inhibition of NFκB, ERK1/2, FAK, AKT, HSP27, Fcer1 and Creb; and a strong inhibition of Jnk, P13KP85, and collagenase type 1 were predicted in the AuHS secretome prepared at 96 h compared to that of 24 h (Figure 6). Notably, ERK1/2 is involved in liver regeneration[38,39] and schwann cell proliferation;[40] Creb plays a positive role in liver regeneration;[41] AKT and FAK are involved in muscle regeneration;[42,43] PI3K and AKT accelerates axonal regeneration,[44] as well as they play important roles in mesenchymal stem cells survival, proliferation, migration, angiogenesis, cytokine production and differentiation.[45] A mild activation of MEK and Nr1 h, and a strong activation of MAPK and CD3 were predicted in the AuHS secretomes prepared at 96 h.

Therefore, the AuHS secretome prepared at 24 h could be more beneficial to be used in regenerative therapy compared to that prepared at 96 h. However, further investigation is required to identify more specific molecular activation and inhibition of the identified functional molecular networks to propagate stem cells to be used for regenerative therapy.

5 | CONCLUSION

The AuHS supplement was shown to favour longer cell viability, and synthesis of more regenerative paracrine factors such as SDF-1A. In addition, HGF and VEGF-A which are involved in angiogenesis, cell proliferation, inhibition of apoptosis and immunoregulation were upregulated in the AuHS secretome. Thus, this study demonstrated that the regenerative potential of the secretome prepared with AuHS supplementation is higher.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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